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Apoptotic Effects of Over-Expressed Estrogen Receptor-β on LoVo Colon Cancer Cell is Mediated by p53 Signalings in a Ligand-Dependent Manner

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Abstract

Epidemiologic studies reported that the prevalence of hereditary non-polyposis colon cancer (HNPCC) in male is about 1.5-fold higher than that in female. Decreases in circulatory estradiol (E_2) have been reported to downregulate the expression of E_2 receptor (ER) and significantly increase the risk of colorectal cancer. Patients that received E_2 replacement therapy were found to have a reduction in the incidence of colon adenoma and carcinoma. Furthermore, significant decreases in the expression of ER have been found in colorectal cancer specimens. These data strongly suggest the protective roles of E_2 and ER against colorectal cancer. However, the mechanisms remain unexplored. LoVo cells were transient transfected to overexpress ER-β, DNA fragmentation and caspase activity assay were performed to evaluate apoptotic effects. Western blotting was used to evaluate protein levels, and luciferase activity assay to measure the TNF-α promoter activity. Our data clearly demonstrated that E_2 and ER-β alone could upregulate p21 and p27 proteins, which further activate caspase-8 and caspase-9 to induce apoptosis in LoVo cell, and the ER-β effects were enhanced by E_2. However, overexpressed ER-β did not influence the expression and promoter activity of TNF-α. In addition, E_2 and overexpressed ER-β downregulated the β-catenin proteins which cause the downregulation of its target genes, cyclin D1 and Rb, to inhibit the cell cycle and cell proliferation. The results indicate that overexpressed ER-β may induce LoVo cell apoptosis and anti-proliferation by increasing p53 signaling in a ligand-dependent manner, and without hTNF-α involvement. Efforts aiming at enhancing ER-β expression and/or activity may prove to be an attractive alternative therapy against colorectal cancer.

Key Words: estrogen receptor-β; LoVo cells; apoptotic effects; antiproliferation

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Introduction

Colon cancer is one of the most common malignancies among populations in the US and Western Europe, and one of the leading causes of worldwide morbidity and mortality due to cancer. In the US, approximately 140,000 new cases and 50,000 deaths are registered each year (9). In Europe, each year about 213,000 new cases and 110,000 deaths are reported, respectively (34).

Unchecked Wnt signaling (36) and/or the loss of cell-cell adhesion (5, 35, 39) are involved in cancer induction and progression. A key event in cancer is the loss of control over β-catenin levels, which can be the consequence of loss-of-function mutations in APC, originally discovered because they predispose to colorectal cancer (36). In addition, activating mutations in β-catenin, of the kind that makes the molecule refractive to downregulation by the APC-axin-GSK-3 β destruction complex, are characteristic of some cancers (36). In colorectal carcinoma, alteration of any component in this complex lets β-catenin escape from degradation, and β-catenin, as a transcriptional factor, activates a variety of genes, such as *c-myc and cyclin D1*, as well as *matrilysin and c-jun*, (4, 11, 25, 41) resulting in increased cell proliferation, decreased apoptosis, and enhanced infiltration capacity (8).

There is no doubt about the role of p53 mutations in the progression of colorectal tumors. The p53 protein is important in maintaining DNA integrity. DNA damage results in p53 and its downstream genes, p21/p27, mediated arrest in G1 phase of the cell cycle, followed by repair or, if the damage is too great, p53 induced apoptosis (15). Both over-expression of exogenous p53, and DNA-damaging agents which activate endogenous p53 resulted in reduced β-catenin in wild-type p53-containing tumor cell lines. The degradation signal controlling β-catenin levels is inducible by p53, revealing a link between genotoxic injury responses and β-catenin degradation (26).

Epidemiological studies have demonstrated that colorectal cancer incidence and mortality rates are lower in women than men (43). Many studies indicate that estrogen replacement therapy (ERT) exerts a protective role against colon cancer in postmenopausal women (17). The effects of estrogen are mediated by two specific, high affinity estrogen receptors, ERα and ERβ, ligand-activated or ligand-independent transcription factors which modulate gene expression by interaction with promoter response elements or other transcription factors (20, 29). Although both receptors have been identified in breast, bone, cardiovascular tissue, the urogenital tract and the central nervous system, ERβ is the form that predominates in the gastrointestinal tract (10, 2). Foley *et al*. showed that malignant transformation in the colon is associated with a marked diminution of ERβ protein expression (7).

Multiple variant forms of ERα and ERβ mRNAs have been identified (28, 24, 45). These show differential expression in human tissues suggesting different functions and may explain the heterogeneous response of tissues to 17β-estradiol (E_2). Contributory roles for ERβ in the regulation of cell differentiation in osteoblast cells (1) and in the induction of apoptosis in neuronal cells (31) have been documented, where it is suggested that E_2 can function as a survival agent or an inducer of apoptosis depending on the ER subtype present in cells. A progressive loss of ERβ expression during the process of carcinogenesis has also been documented in prostate (37) and breast cancers (14), suggesting a role for ERβ as a potential inhibitor of cellular proliferation and/or transformation. In colon cancer research, the downregulation of the ERβ mRNA has been reported to easily lead to the cancer production (2) and ERβ has been found to prevent the formation of cancer (19).

The primary aim of present study was to determine whether, and if so how, E_2 and ERβ protects against colon cancer, we have assessed the effects of E_2 and ERβ on apoptosis in the LoVo colon cancer cell line, by using transient transfected to overexpress ERβ. Moreover, we expect to unveil the roles of p53 signaling pathway and β-catenin in the E_2 and ERβ effects.

Materials and Methods

Materials

Human colon cancer cell lines, LoVo, were obtained from the Global Bioresource Center (ATCC, Manassas, VA, USA). LoVo cells were established from the metastatic nodule resected from a 56-year-old colon adenocarcinoma patient.

Cell Culture and Transfection

LoVo cells were maintained in RPMI 1640 medium (R-6504, Sigma) augmented with 1.5 g/l sodium bicarbonate, and 10% fetal bovine serum (Hyclone, UT, USA) in a humid atmosphere at 37°C, with 5% CO_2. Cells were passaged twice a week, for example, on DNA fragmentation and luciferase assay, the cells were transferred to 6-cm culture dishes and allowed to grow to 60-70% confluency. Transfection were carried out using the liposome (Invitrogen Lipofectamine 2000 transfection kit) with phenol-red free, serum-free medium RPMI 1640 (Sigma, St. Louis, MO, USA), followed by treatment with E_2, for 12-16 h. Cells seeded at 60-mm dish were cultured for 24 h in RPMI 1640 supplemented with 10% FBS, rinsed with

serum-free RPMI 1640 and 1 ml RPMI 1640 containing 15 μg/ml liposome, and 2 or 4 μg pcDNA. The cells were incubated at 37°C for 6 h before adding 1 ml RPMI 1640 supplemented with 10% FBS to the medium. After incubation for 18 h, the medium was replaced with fresh 10% FBS-RPMI 1640.

Construction of Plasmid

The human estrogen receptor β (hER-β) was generated by PCR from pRST7-ERβ plasmid, and the PCR product was inserted into *Bam* HI and *Sal* I sites in the pTRE2-hygromycin B vector (Promega Corp., Madison, WI, USA). The insert was verified by DNA sequencing.

Isolation of Apoptotic DNA Fragments

Isolation of apoptotic DNA fragments was performed according to Herrmann *et al*. (12). After harvesting, the cells were washed twice with phosphate buffered saline (PBS) and centrifuged at 12,000 rpm for 5 min. The cell pellets were resuspended in lysing buffer (pH 7.5) containing 1% NP40 in 20 mM EDTA and 50 mM Tris-HCl and centrifuged at 15000 rpm for 5 min. After adding 1% sodium dodecyl sulfate (SDS) and RNase-A (Roche, Indianapolis, USA) (final concentration 3.33 μg/μl), the supernatants were incubated at 56°C for 2 h and digested with proteinase K (Sigma, St. Louis, MO, USA) (final concentration of 2.5 μg/μl) at 37°C for 2 h. DNA was then precipitated by adding a mixture of 1/2 vol. Ten M ammonium acetate and 2.5 vol. ethanol, dissolved in loading buffer, separated by electrophoresis in 1.5% agarose gel, and stained with ethidium bromide.

Western Blotting

Cell harvesting, proteins were extracted using lysis buffer (50 mM Tris-base, 0.5 M NaCl , 1.0 mM EDTA, 1% NP40, 1% glycerol, 1 mM -mercaptoethanol, proteinase k inhibitor), centrifugating to discard the pellet. The protein concentrations were determined by the Lorry assay. The proteins of cell lysates were analyzed by SDS-PAGE, and proteins were transferred to nitrocellulose (or PVDF). Residual protein sites on nitrocellulose papers were incubated at room temperature for 2 h and then immerse in blocking buffer containing 5% skim milk in Tween/Tris-buffer saline (TBS). After washing with buffer, the nitrocellulose papers were immerse in monoclonal antibodies of estrogen receptor-β (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), α-tubulin (NeoMarkers, Fremont, CA, USA) Caspase3, Caspase8, Caspase9 (Santa Cruz) diluted with TBS containing 5% skim milk and incubated at room temperature for 4 h. The immunoblots were then washed in triplicate with TBS for 10 min before immersing in the second antibody solution for 1 h and diluted 1000-fold in TBS. The filters were then washed in triplicate in TBS for 10 min. Antibody reaction was visualized with chemiluminescence reagent for Western blotting.

Luciferase Assay

Transient transfections of pGal-hTNF-α-Luc were performed using the QIAGEN plasmid Maxi Kit. Transfections were performed according to instructions of manufacturer. The transfections were repeated at least twice to ensure reproducibility of the results. The transfection with pGal-ERE-Luc was used as a positive control. Activities of Luciferase were measured using the dual reporter gene system (Promega, San Luis Obispo, CA, USA) with an automated chemiluminescence detector (Berthold, Bad Wildbad, Black Forest, Germany).

Statistical Analysis

All data are expressed percentages of the control and mean ± S.D. The results were based on three independent experiments. Student's unpaired *t* test was used to compare the differences between groups. Experimental group *vs*. control group: A P value < 0.05 was considered statistical significance, $*P < 0.05$ and $**P < 0.01$.

Results

Efficiency of Transiently Transfected ERβ in LoVo Colon Cancer Cell Line

Transient transfection in LoVo colon cancer cell line was carried out by using Lipofectamine to overexpress ERβ, and the protein level was indeed higher in ERβ transfectant than empty vector, pcDNA3 transfectant (Fig. 1a). The efficiency of ERβ transfectant was evidenced by transfecting plasmids that contain GFP gene and visualizing with fluorescence microscopy. The efficiency was up to 40% (Fig. 1b).

Apoptotic DNA Fragments in ERβ Ttransfectant after E_2 Treatment

The yields of apoptotic DNA fragments in wild type ERβ transfectant was significantly higher in the group treated with E_2 (10^{-8} M) than that with ethanol (Fig. 2: Lanes 3 and 4). Similar findings were also in the pcDNA3 transfectant (Fig. 2: Lanes 1 and 2). In addition, the ERβ transfectant had a significant higher yield of apoptotic DNA fragments than pcDNA3 alone after E_2 treatment (Fig. 2: Lanes 2 and 4).

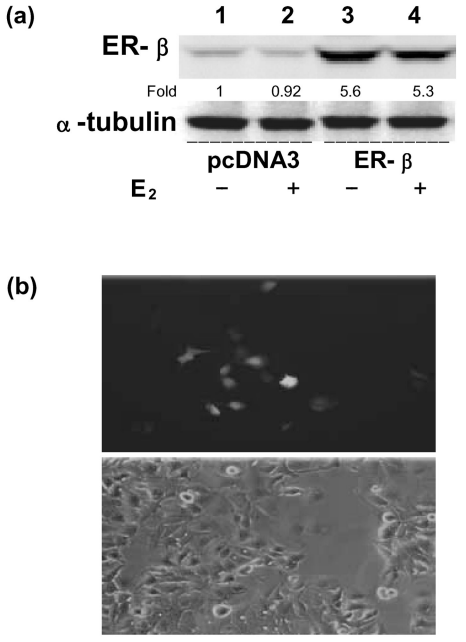

Fig. 1. Efficiency of transiently transfected ERβ in LoVo colon cancer cell line. (a) Protein contents in the ERβ and pDNA3 transfectants. LoVo cells transfected with pcDNA3 (2 μg) or ERβ (2 μg) after treatment with estradiol (E_2). Lane 1: pcDNA3 transfectant treated with ethanol. Lane 2: pcDNA3 transfectant treated with E_2 (10^{-8} M). Lane 3: ERβ transfectant without E_2 treatment. Lane 4: ERβ transfectant treated with E_2 (10^{-8} M). (b) LoVo cells transfected with GFP plasmid and visualized with fluorescence microscopy (upper panel), visualized with optical microscopy as control (lower panel). This is a representive of two independent experiments with similar results.

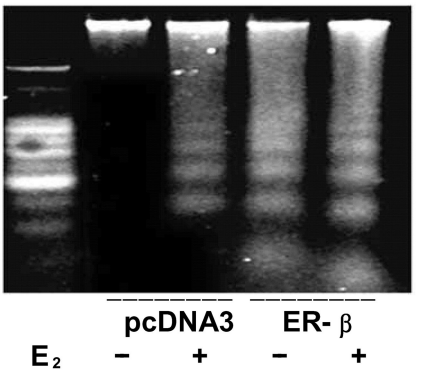

Fig. 2. Apoptotic effects determined by DNA fragmentation. Isolation of apoptotic DNA fragments from the LoVo cells transfected with pcDNA3 (2 μg) or ERβ (2 μg) after treatment with estradiol (E_2). Lane 1: pcDNA3 transfectant treated with ethanol. Lane 2: pcDNA3 transfectant treated with E_2 (10^{-8} M). Lane 3: ERβ transfectant without E_2 treatment. Lane 4: ERβ transfectant treated with E_2 (10^{-8} M). DNA fragments were isolated by electrophoresis in 1.5% agarose gels, and stained by ethidium bromide. This is a representive of two independent experiments with similar results.

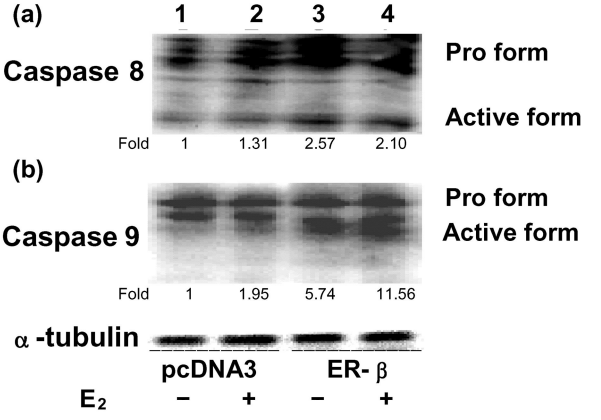

Fig. 3. Activation of caspases in LoVo cells transfected with pcDNA3 or ERβ after estradiol (E_2) treatment. (a) Levels of pro-form and activated form caspase 8 in the two transfectants after estradiol (E_2) treatment. (b) Levels of pro-form and activated form caspase 9 in the two transfectants after estradiol (E_2) treatment. Preparations treated with ethanol were employed as controls and α-tubulin as internal controls. This is a representive of two independent experiments with similar results.

Changes in the Procaspases and Cleavaged Caspases in ERβ Transfectant

Protein contents of procaspases were significantly higher in ERβ transfectant than the pcDNA3 transfectant. The active forms of caspase 8 and caspase 9 increased in the ERβ transfectant and after E_2 treatment even more higher increase (Fig. 3a, b). It suggests ERβ alone could induce caspase 8/9 activation and lead to apoptosis, which augmented by E_2 treatment.

TNF-α Promoter Activity Was Not Activated by E_2 and/or ERβ Overexpressed in LoVo Cells

The activation of caspases is passed by two main pathways: mitochondrial pathway and the death ligands and death receptors (30). To determine whether ERβ induced caspase activation through TNF-α signaling pathway, we employed luciferase assay. After transfecting both ERβ and pGL3-ERE-Luc into the LoVo cells, the expression of luciferase increased by 20 folds. In the presence of E_2, these transfectants showed more increase to 45-fold in luciferase expression (Fig. 4a). However, the expression of luciferase did not change in both ERβ and pGL3-hTNF-α-Luc co-transfected with/without E_2 treatment (Fig. 4b). In addition, no protein content change of TNF-α under ERβ transfected with/without E_2 treatment (Fig. 4c).

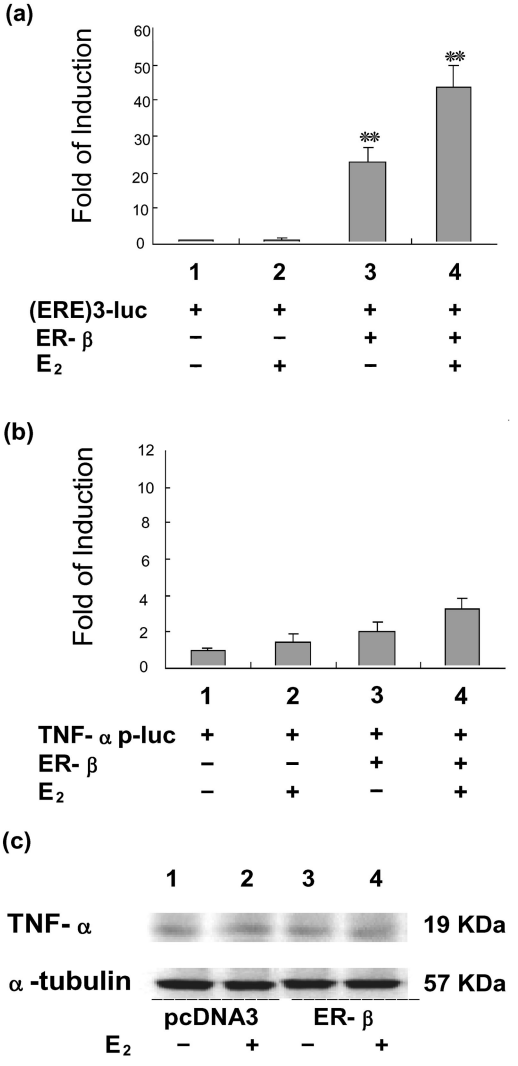

Fig. 4. TNF-α promoter activity after overexpressing ERβ with E_2 in LoVo cells. (a) LoVo cells were transiently co-transfected with pcDNA3 or ERβ with ERE-tk-α-Luc and treated with either ethanol or E_2. (b) LoVo cells were transiently co-transfected with pcDNA3 or ERβ with pGL3-hTNF-α-Luc and then treated with either ethanol or E_2. TNF-α promoter gene activity was measured by Luciferase assay. All treatments of each bar are described as indicated. The ERE-Luc activities were used as positive control. (c) Protein expression of TNF-α in the ERβ and pDNA3 transfectants. These results were based on three independent experiments. **Significantly different compared with the control group ($P < 0.01$).

Changes in the P53 Signaling Proteins in ERβ Transfectant

In the group of ERβ transfectant with E_2 treatment, p21 (3.75-fold), p27 (2.35-fold), and their upstream p53 (1.93-fold) protein level increased significantly (Fig. 5). Protein contents of Cyclin D1 (0.52-fold), Cyclin E (0.36-fold), and Rb (0.57-fold) which control cell cycle

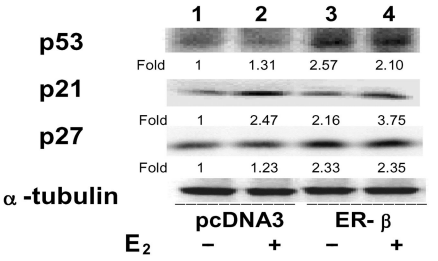

Fig. 5. Changes of the p53 signaling proteins in ERβ overexpressing LoVo cells. Protein levels of the p53 and it's downstream p21 and p27 were measured by Western blotting, α-tubulin was used as internal control. This is a representive of two independent experiments with similar results.

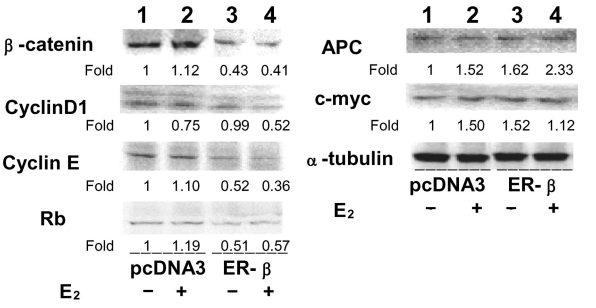

Fig. 6. Changes of the regulatory genes in cell cycle and Wnt signalling pathway in ERβ overexpressing LoVo cells. Protein levels of the cell cycle regulatory genes (cyclin D1, cyclin E, Rb, and c-myc) and Wnt pathway relative proteins (APC and β-catenin) were analyzed by Western blotting, α-tubulin was used as internal control. This is a representive of two independent experiments with similar results.

progression from G1 phase into S phase decreased significantly in ERβ transfectant with E_2 treatment (Fig. 6). It suggests E_2 binding to ERβ could suppress colon cancer cell proliferation and induce apoptosis.

Changes in Wnt-APC-β-Catenin Signalling Pathway in Colon Cancer

Protein content of β-catenin decreased significantly in ERβ transfectant with E_2 treatment, corresponding with the change of Cyclin D1 (0.52-fold) and Rb (0.57-fold), the downstream target gene of β-catenin (0.41-fold) (Fig. 6). The result might indicate that ERβ with E_2 in colon cancer could inhibit cell proliferation, migration, and carcinogenesis.

Discussion

Here provides evidence that ERβ play a role to

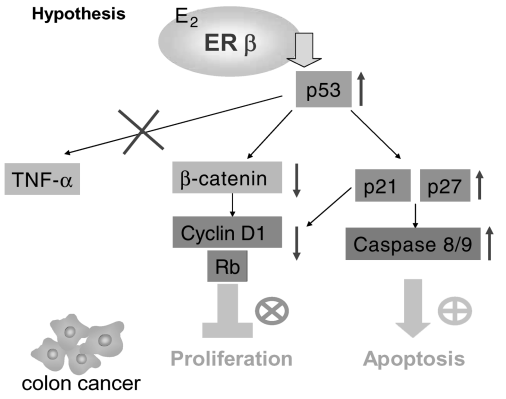

Fig. 7. A depicted flow chart of overexpressed ERβ may induce apoptosis and inhibit proliferation of LoVo cells by activating p53, which either activate p21/p27, moreover activate caspase 8/caspase 9 or suppress cyclin D1/cyclin E and lead to apoptosis and cell cycle arrest, or degrade β-catenin and downregulate cyclin D1/E and Rb, then inhibit cell proliferation.

engage apoptosis and anti-proliferation in LoVo colon cancer cells. Overexpressed ERβ proteins after transiently transfecting the ERβ (Fig. 1), the caspase 8/9 were activated leading to apoptosis; β-catenin, cyclin D1/E, Rb proteins decreased leading to inhibiting cell cycle progression, and cell proliferation (Fig. 6). It suggested ERβ maybe a good candidate to suppress colon cancer, and moreover for clinical therapy.

The executioner caspases (aspartate-specific cysteine protease) is main pathway orchestrating the programmed cell death (38). The caspase gene family contains 14 mammalian members and there are 11 identified human enzymes. This family is subdivided into two major phylogenic subfamilies: the inflammation group (caspase-1, -4, -5, and -13) and the apoptosis group (caspase-2, -3, -6, -7, -8, -9, and -10) (27, 41). The activation of caspase is passed by two main pathways: mitochondrial pathway and the death ligands and death receptors (30). Since apoptosis is essential for development and maintenance of multicellular organisms, a deficiency in apoptosis is one of the key events in pathophysiology of tumor development (46). We found that ERβ with E_2 induced apoptosis in DNA fragmentation assay, and activated caspase 8 and caspase 9 in Western blotting assay alone with p53 elevation. In hepatoma cell line, caspase 8 is a target gene of p53 (22), and caspase 9 is required for p53-dependent apoptosis and chemo sensitivity in a human ovarian cancer cell line (44). However, TNF-α, the death receptors signaling pathway, can induce colon cancer apoptosis had been demonstrated (18, 42), but in our experiment, TNF-α did not mediate ERβ induced caspase activation, as evidenced by TNF-α reporter gene activity and TNF-α protein expression (Fig. 4).

Genistein, a soy metabolite that is a selective agonist of ERβ, has been shown to induce p21WAF1/CIP1 expression in various human cancers including colorectal carcinoma, thus leading to cell-cycle arrest (21, 32). Cyclin D1 is not expressed in normal colorectal tissue, but deregulated expression of cyclin D1 is well documented in colorectal cancers (13, 33). In addition, ERβ interferes with this pathway of carcinogenesis by inhibiting cyclin D1 gene transcription (23). These finding correspond with ours, that ERβ with E_2 induced not only p21 and p27 but also p53 protein expression, and suppressed cyclin D1, cyclin E and Rb protein expression, leading to interference cell cycle and proliferation.

When APC are mutated, resulting constitutive activation of the Wnt pathway provides a strong selective advantage by affecting cell proliferation, migration, apoptosis and possibly differentiation of the intestinal stem cell (6). β-catenin is the central player, which is a transcription cofactor with T cell factor/lymphoid enhancer factor TCF/LEF in the Wnt pathway (3) and a structural adaptor protein linking cadherins to the actin cytoskeleton in cell-cell adhesion (16). We found the protein content of β-catenin decreased significantly in ERβ transfectant with E_2 treatment, corresponding with it's downstream target gene, cyclin D1 and Rb.

Taken together, our data suggests ERβ with E_2 activate p53 pathway, might leading to activate p21, p27, and degrade β-catenin, which results in caspases 8/9 activation and cyclin D1/E, and Rb inhibition, thus providing apoptotic activation and proliferating inhibition. We would like to further explore the effects with ERβ on invasion and migration of colon cancer and the relative mechanism. Efforts aiming at enhancing ER-β expression and/or activity may prove to be an attractive alternative therapy against colorectal cancer.

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